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3. Donnelly, J.J., Ulmer, J.B., and Liu, M.A. Immunization with DNA. Journal of Immunological Methods vol. 176 no.2, pp 145-152 (1994)

4. Thomas, L.J., Pickard, M.D., Stewart, S.E., Waite, B.C.D., Lin, A.Y., Rittershaus, C.W., Pettey, C.L. A plasmid-based vaccine to elicit autoantibodies to cholesteryl ester transfer protein (CETP) for the prevention/treatment of atherosclerosis. Journal of Allergy and Clinical Immunology 99 (1 part 2):pS187 (1997)

5. Kyoo, Jang Moon, Byung-Yoon, Ahn, Tae-Lin, Huh, Song-Hae, Bok, Bok, Park Yong, Expression of cholesteryl ester transfer protein cDNA using recombinant vaccinia viruses. Journal of Biochemistry and Molecular Biology vol 28 no. 3, pp216-200 (1995).

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Review article

Immunization with DNA

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1. Introduction

Although the ability of live virus vaccines to produce effective immunity has long been known, the multiple pathways of antigen processing that provide the biological basis for the ability of virus infections to elicit MHC class I-restricted cell-mediated immunity were elucidated only relatively recently. This understanding led to the consideration of direct transfection of tissue cells in vivo as an alternative means of obtaining appropriate antigen processing, and thus as an alternative to immunization with live viruses. The ability of direct injection of non-replicating plasmid DNA coding for viral proteins to elicit protective immune responses in laboratory and preclinical models has suggested that immunization with DNA might be clinically useful for the vaccination of humans. Both antibody and cell-mediated immune responses have been demonstrated, indicating that DNA vaccination can serve as an alternative to immunization with live attenuated viruses, and thus may provide improved protection relative to killed virus vaccines.

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2. Immune responses evoked by administration of polynucleotides in vivo

In models of viral disease where challenge of immunized animals is possible, both antibodies and cell-mediated immune responses induced by direct inoculation with plasmid DNA encoding viral proteins in vivo have been found to be protective. Antibodies have been raised in various species including mice, ferrets, cattle, and non-human primates by the intramuscular injection of plasmid DNAs that encoded the hemagglutinin (HA), matrix protein, and nucleoprotein (NP) from influenza A virus (Ulmer et al., 1993; Montgomery et al., 1993; Donnelly et al., 1994), gp120 (Rhodes et al., 1994) and gp160 (Wang et al., 1993a, b) from HIV, gIV from bovine herpesvirus I (Cox et al., 1993), surface glycoprotein from rabies virus (Xiang et al., 1994), and hepatitis B surface antigen (Davis et al., 1993b). Virus neutralizing activity was demonstrated in the antibodies raised against influenza HA for homologous strains of influenza (Montgomery et al., 1993), the anti-gp160 antibodies for homologous strains of HIV (Wang et al., 1993a, b), and the antibodies to rabies surface glycoprotein for rabies virus (Xiang et al., 1994). Since the epitopes of influenza HA that are recognized by hemag-

glutination inhibiting (HI) and neutralizing antibodies are composed of non-contiguous regions of the HA polypeptide (Wiley et al., 1981), the ability to generate these antibodies indicates that the HA is present in its native conformation in or on the transfected cells. The antibody responses induced by intramuscular injection of DNA persisted for 6 months or more in African Green monkeys and for 13 months or more in mice and Rhesus monkeys (Yankauckas et al., 1993; Donnelly et al., unpublished). Intramuscular injection of plasmid DNA that coded for the HA from A/PR/8/34 (H1N1) provided complete protection from disease when mice were given a lethal homologous challenge with A/PR/8/34 (Montgomery et al., 1993), and ferrets that were immunized with HA DNA from the A/PR/8/34 strain or the recent clinical isolate A/Georgia/03/93 (H3N2) had significantly reduced viral shedding upon challenge with the homologous strain of influenza (Donnelly et al., 1994, and unpublished). Similarly, mice that had been immunized with the rabies virus glycoprotein by intramuscular injection of DNA were protected from death following challenge with 5 LD_{50} of rabies virus (Xiang et al., 1994). Injection of chickens with HA DNA from A/Seal/Mass/1/80 (H7N7) intramuscularly or by a combination of intramuscular, intravenous and subcutaneous routes provided protection against death upon challenge with a lethal strain of avian influenza virus (A/Chicken/Victoria/1/85, H7N7) (Robinson et al., 1993). Intramuscular injection of DNA coding for the gIV of bovine herpesvirus likewise protected cattle against challenge (Cox et al., 1993).

The ability of gold microprojectiles, coated with DNA and propelled into the epidermis at high velocity, to transfect epidermal cells and thereby induce immune responses to the foreign proteins expressed, was demonstrated initially by Tang et al. (1992). Immunization of mice by this method of biolistic transfection has induced antibodies against human growth hormone (Tang et al., 1992), influenza HA, and HIV gp120 (Eisenbraun et al., 1993). Immunization of chickens with influenza HA DNA by biolistic transfection caused a reduction in mortality after challenge with a homosubtypic strain of influenza (Fynan et al., 1993). In mice, delivery of A/PR/8/34 HA DNA by this method also induced a protective immune response against subsequent homologous challenge with A/PR/8/34 (Fynan et al., 1993; Eisenbraun et al., 1993). Splenic B cells from mice that were immunized against human growth hormone by this method were used to generate monoclonal antibodies that reacted with both native and denatured forms of human growth hormone (Barry et al., 1994).

Intramuscular injection of mice with DNA plasmids encoding antigen genes also induces cytotoxic T lymphocytes (CTL), whose effector function can be demonstrated upon restimulation of spleen or lymph node cells with antigen, or mitogen and IL-2, in vitro. Effectors from mice immunized with DNA that encoded the NP from influenza A virus were capable of lysing target cells infected with influenza virus, as well as target cells that were pulsed with peptide epitopes appropriate to the H-2 restriction element (Ulmer et al., 1993; Donnelly et al., 1994). The CTL against influenza NP were induced by a single intramuscular injection of as little as 1 μg of NP DNA (Ulmer et al., 1994). In other studies, CTL were found to persist for more than 1 year after immunization (Yankauckas et al., 1993; Donnelly et al., unpublished). Mice immunized intramuscularly with DNA coding for NP that was cloned from the A/PR/8/34 (H1N1) strain of influenza were protected from death and weight loss following challenge with an influenza strain of a different subtype (A/Hong Kong/68, H3N2) that was isolated 34 years later (Ulmer et al., 1993). Likewise, CTL have been generated in mice that recognize the glycoprotein of rabies virus (Xiang et al., 1994). Immunization of ferrets with the same plasmid elicited an immune response that significantly reduced viral shedding in nasal washes 5–6 days after challenge with the A/PR/8/34 and A/HK/68 strains (Donnelly et al., 1994). Ferrets that were immunized with DNA coding for the NP and matrix (M1) from the A/Beijing/353/89 (H3N2) strain had significantly reduced viral shedding upon challenge with the antigenic drift variant A/Georgia/03/93 (Donnelly et al., unpublished). Combined immu-

nization of ferrets with the NP and M1 genes from A/Beijing/353/89 and the HA from the closely related strain A/Hawaii/01/91 provided protection of ferrets against the A/Georgia/03/93 drift variant that was equivalent to the protection achieved by immunization with homologous A/Georgia/03/93 HA DNA (Donnelly et al., unpublished). These results illustrate the utility of immunization with cDNA vectors encoding proteins from influenza, both to generate protective antibody responses and to induce cellular immunity that is able to confer cross-strain protection.

Antitumor immune responses have been elicited by direct injection of plasmid DNA that encoded antigenic cell surface proteins in mice. BALB/c mice were protected from subsequent challenge with a syngeneic myeloma line (SP2/0) that had been transfected with the HIV gp160 or human CD4 genes by immunization with plasmids that encoded the gp160 from HIV, or human CD4, respectively (Wang et al., 1994). This protection may be mediated by ADCC directed against antigens expressed on the surface of the transfected cells. Although this study used tumor cells, it also illustrated the potential utility of immune responses against HIV evoked by DNA, which cannot be tested directly in a mouse model. Injection of plasmid DNA that encoded MHC class I antigens, complexed with lipofectin, into tumors in mice induced an immune response that arrested the growth of the tumors and caused complete tumor regression in some instances (Plautz et al., 1993). Nabel et al. (1993) induced CTL that recognized autologous tumor cells after intratumoral injection of HLA-B7 DNA in humans lacking this MHC antigen, and therefore capable of mounting an alloimmune response. Mice also have been immunized with human carcinoembryonic antigen (CEA) as a potential therapy for CEA-expressing carcinomas (Conry et al., 1994). Polynucleotide vaccination could be used to develop individualized anti-idiotype vaccines against human B cell lymphomas, using V regions cloned from individual tumors by PCR (Hawkins et al., 1993). Immunization of mice with a construct encoding the V region of a human κ light chain induced antibodies that reacted with a human IgM myeloma protein which contained the same light chain (Watanabe et al., 1993). Anti-idiotypic antibody responses have been elicited in mice against the B1.8 hybridoma and the BCL1 B cell lymphoma, using plasmid DNA that encoded the V genes of the tumor-specific idiotype Ig (Hawkins et al., 1993).

Alloimmune responses also have been induced by both biolistic and intramuscular immunization. Intramuscular injection of DNA coding for the RT1.A^a MHC class I molecule in Lewis (RT1.A^l) rats resulted in accelerated rejection of RT1.A^a cardiac allografts, and increased alloantibody and RT1.A^a-specific CTL responses after transplantation (Geissler et al., 1993). AKR/J (H-2^k) mice given biolistic transfections with a plasmid encoding the a chain of the H-$2K^b$ MHC class I antigen produced CTL capable of killing H-$2K^b$ targets (Hui et al., 1994). Muscle cells from Lewis rats also were transfected with the RT1.A^a MHC antigen and implanted in the thymus of Lewis recipients, which were then grafted with ACI (RT1.A^a) liver and treated with antilymphocyte serum (ALS) (Knechtle et al., 1994). The mean survival time of liver allografts in treated rats was 83 days, a significant increase compared with 26.7 days in sham-treated rats given control muscle cells plus ALS (Knechtle et al., 1994). Thus, like endogenous MHC molecules, alloantigens expressed in vivo on transfected muscle cells can deliver immunogenic or tolerogenic signals depending on the site of expression.

3. Methods of inducing immune responses with DNA

Transfection of mammalian cells in vivo by injection of DNA was observed as early as 1960 by Ito (1960, 1961), who were able to induce papillomas in rabbit skin by injecting phenol-extracted nucleic acids from Shope rabbit papilloma virus. These observations on papilloma virus were later extended by Brandsma et al. (1991). Nicolau et al. (1983) obtained reductions in blood glucose suggesting the overexpression of insulin following injection of preproinsulin DNA in liposomes, while Benvenisty and Reshef (1986) obtained ex-

pression of chloramphenicol acetyl transferase (CAT), hepatitis B virus surface antigen (HBsAg), human growth hormone, and insulin by intraperitoneal injection of calcium phosphate-precipitated DNA. Letvin et al. (1991) reported that intramuscular injection of bacteriophage lambda DNA containing an SIV_{MAC} proviral DNA in cynomolgus monkeys (*M. fascicularis*) could induce seroconversion and production of intact SIV_{MAC} virus. Transfection with DNA in vivo was demonstrated directly by Wolff et al. (1990), using intramuscular injection, and by Williams et al. (1991) using the biolistic approach.

Direct intramuscular injection of plasmid DNA has been relatively widely used to induce immune responses, due to its simplicity and effectiveness. Wolff et al. (1992) observed that physiological saline solution appeared to be the optimal vehicle for expression of reporter genes in mouse skeletal muscle, but that implantation of calcium phosphate-precipitated DNA also could be effective. Manthorpe et al. (1993) evaluated the efficiency of a variety of promoters including CMV, RSV, SRa, actin, MCK, α-globin, adenovirus, and dihydrofolate reductase for muscle transfection and found that the CMV and RSV promoters gave the highest levels of expression of firefly luciferase. Addition of other components such as Tris-EDTA to the saline vehicle neither increased nor reduced its effectiveness by a statistically significant amount, except for hydroxychloroquine and a cationic peptide (PKKKRKVEDPYC), which interfered with reporter gene expression (Manthorpe et al., 1993). Injection volumes of 12–100 μl per rectus femoris muscle were fully effective, with 50 μl per muscle being commonly used as a convenient volume for mouse immunization studies. Reporter gene expression reached a maximum during the first 60 days and then declined slightly thereafter; detectable levels of reporter gene expression were still present 1.5 years after injection of DNA (Wolff et al., 1992). Protective levels of both HI antibodies and CTL that were effective against homologous and cross-strain challenges, respectively, were induced by three intramuscular injections of 1 μg of plasmid DNA in saline (Ulmer et al., 1994). Wells and Goldspink (1992) suggested that muscle growth, as occurs for example in young and rapidly growing mice, could increase reporter gene expression; however, subsequent studies in our laboratory have shown that plasmid DNA could elicit protective immune responses in mature mice at ages of at 1 year or more (Ulmer et al., unpublished). Wells and Goldspink (1992) also observed differences in reporter gene expression between male and female mice, perhaps as a result of different growth kinetics between the sexes. In our laboratory we compared the immune responses induced by DNA injection in male and female mice and found that they were equivalent (Ulmer et al., unpublished). Davis et al. (1993a) also suggested that muscle that was made edematous, by intramuscular injection of hypertonic 25% sucrose solution 20 min prior to administration of DNA in mice, gave increased reporter gene expression. However we did not observe a significant enhancement of antibody responses in Rhesus monkeys by this method (Donnelly et al., unpublished); such an approach may not be as feasible for use in humans as it would be in laboratory animals.

Administration of toxic agents intended to cause muscle necrosis and repair, either prior to or concurrently with injection of DNA, has been used by some investigators to increase expression. Wang et al. (1993a) used an injection of 50 μl of the local anesthetic, bupivicaine, administered 24 h prior to injection of the DNA at a concentration of 0.5%. Bupivicaine pretreatment (0.5 ml of a 0.5% solution, administered 24 h before injection of the DNA) also has been used in non-human primates to increase expresson of HIV gp160 constructs (Wang et al., 1993b). Wells (1993) reported an increase in CAT activity in transfected mouse tibialis anterior muscles pretreated with 0.5% bupivicaine, and Vitadello et al. (1994) showed an increase in reporter gene expression after bupivicaine pretreatment of rat soleus muscles. Davis et al. (1993b) used 50 μl of 10 μM cardiotoxin, administered 5 days prior to injection of the DNA, to induce one cycle of muscle necrosis and repair. This was thought to result in an increase in expression that was manifested as an earlier antibody response to HBsAg.

Intradermal injection of plasmid DNA, by nee-

dle injection rather than by the biolistic device, also has been used in some laboratories. Fynan et al. (1993) reported that intradermal injection of A/PR/8/34 HA DNA in mice induced a protective response against homologous challenge with influenza, although with slightly less efficacy than intramuscular administration. Yankauckas et al. (1993) reported that intradermal injection of DNA encoding influenza NP was able to induce antibodies and CTL against NP and a protective immune response. However, studies in our laboratory in mice injected intradermally or intramuscularly with NP DNA suggested that, although antibodies and CTL could be induced by this method, the protective efficacy against a cross-strain challenge with A/HK/68 (H3N2) was less than that of intramuscular injection of an equal amount of DNA (Fig. 1). Intravenous administration of naked DNA has been reported by some investigators to induce protective responses against influenza challenge in mice (Fynan et al., 1993); studies in our laboratory indicated that weak CTL responses and variable antibody responses were induced by this route of administration compared with intramuscular injection. Intravenous administration of DNA complexed with lipofectin was reported by Zhu et al. (1993) to result in expression of reporter genes in a variety of tissues. Transfection of bronchial epithelial cells by aerosolization (Stribling et al., 1992) and of T lymphocytes by i.p. injection (Philip et al., 1993) of DNA:lipofectin complexes also have been reported. Nabel et al. (1993) showed that direct injection of DNA:lipofectin complexes into tumors induced expression of foreign MHC antigens encoded by the injected DNA. Intraperitoneal injection of liposomes containing RNA coding for influenza A NP also has been reported to induce CTL specific for the H-2d-restricted NP147-155 peptide in BALB/c mice and the NP48-66 peptide in C3H mice (Martinon et al., 1993). Thus although uptake and expression of plasmids in skeletal muscle appears to be maximal when uncomplexed DNA is used, other tissue cells which are poorly transfected by 'naked' DNA appear to be more efficiently transfected by DNA in liposomes or associated with cationic lipids.

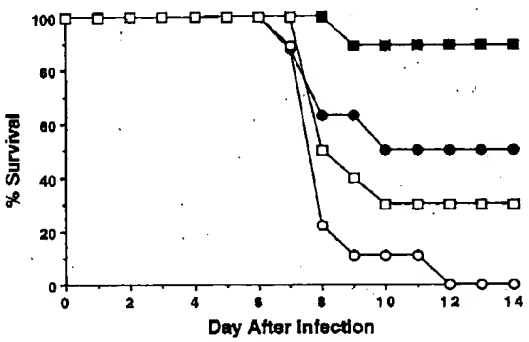

Fig. 1. Comparison of protective immune responses to influenza in mice immunized intradermally and intramuscularly with NP DNA. Groups of ten BALB/c mice were injected at 4, 7 and 10 weeks of age with 100 μg of plasmid DNA encoding the NP from influenza A/PR/8/34, intramuscularly (solid squares) or intradermally (solid circles). Intramuscular injections were given in both quadriceps muscles in a total volume of 100 μl. Intradermal injections were given in five sites of shaved dorsal skin in a total volume of 100 μl. Control mice (open circles) were given three injections of 100 μg of control vector (without an inserted gene) intradermally, or were not injected (open squares). Mice were challenged with 200 $TCID_{50}$ of influenza A/HK/68 at 13 weeks of age. Mice that received NP DNA intramuscularly experienced significantly better survival ($p < 0.0022$ by χ^2) than mice given an equal amount of NP DNA intradermally. Mice that were given NP DNA i.m. experienced significantly better survival than mice either given control vector or not injected ($p < 10^{-6}$). Mice that were given NP DNA i.d. experienced significantly better survival than mice given control vector ($p < 0.000012$ by χ^2), but their survival was not significantly different from that of uninjected mice ($p < 0.93$).

Biolistic transfection with DNA-coated gold microparticles is accomplished by mixing the DNA with gold particles (2–5 μm in diameter) in aqueous solution, and allowing the DNA to adsorb. The particles are then washed with ethanol and dried (Eisenbraun et al., 1993). To deliver the particles to the target tissue, the particles are placed on a stage which is accelerated, either by helium gas under high pressure (Williams et al., 1991; Tang et al., 1992), or by the shock wave created by an electrical discharge (Eisenbraun et al., 1993; Fynan et al., 1993), toward a stationary screen which stops the forward movement of the stage. The particles continue to move at high velocity until they strike the target tissue. Trans-

fection appears to be mediated by direct penetration of cells by the gold particles. Maximal expression of reporter genes was found at μg DNA:mg gold ratios of 0.1–5.0 (Eisenbraun et al., 1993). Expression of reporter genes in transfected mouse skin sites persisted for approximately 6 days, after which expression was lost, apparently due to normal sloughing of the epidermal cells, although some expression may persist in dermal fibroblasts or Langerhans cells (Williams et al., 1991).

4. Potential clinical uses

Potential clinical applications for polynucleotide vaccination are found in many infectious diseases. DNA vaccines can be designed to yield both cell-mediated and humoral immune respones from a single vaccination; this cannot generally be accomplished with non-replicating vaccines. Therefore, viral diseases in which CMI is thought to have an important role in host defense may be amenable to a polynucleotide vaccine approach. Some examples of this are influenza, HSV, and rabies. In the case of HSV and rabies, live attenuated virus vaccines may be less likely to be considered safe, and therefore the polynucleotide vaccine approach might be advantageous for its relative safety as well as its immunogenicity. The utility of DNA vaccination for bacterial diseases remains to be demonstrated. Bacterial diseases in which protein toxins have an important pathogenic role, for example enterotoxigenic staphylococci or *E. coli*, may be ameliorated by the long-lived antibody response than can be induced by PNV. MHC class I-restricted $CD8^+$ T cells may be important in host defense against intracellular pathogens such as *Listeria monocytogenes*, *Mycobacterium* spp., *Leishmania* spp. (Xu and Liew, 1994), and *Plasmodium* spp. (Sedegah et al., 1994), and therefore a polynucleotide vaccine approach might be considered for these diseases. The therapeutic uses of DNA vaccines are beginning to be explored; the ability of direct DNA injection to elicit alloimmune and bystander killing reponses in humans and animals suggests that it may be a useful approach to tumor immunotherapy. As immunotherapeutic approaches to viral diseases such as HIV become better understood, the long-lived immune responses induced by polynucleotide vaccines may prove to be therapeutically useful.

5. Conclusion

Immunization with DNA is a simple, robust, and effective means of eliciting both antibody and cell-mediated immune responses. In selected animal models where challenge studies can be performed, this method of immunization may provide protective immunity that is at least equivalent to that produced by immunization by conventional methods. The extent to which this method can be applied to proteins not of vertebrate origin, e.g., antigens from bacteria and protozoan parsites, remains to be determined. However the present experience indicates that DNA immunization is a useful method of raising immune responses, including monoclonal antibodies, against viral proteins, immunoglobulins, and other antigens of immunological interest.

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